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Substitutions in the Protease ($3C^{pro}$) Gene of Poliovirus Can Suppress a Mutation in the 5′ Noncoding Region

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The poliovirus mutant 5NC-11 has a 4-base insertion at position 70 within the 5′ untranslated region and is deficient in RNA synthesis. Revertants from 5NC-11 were isolated, showing a partial recovery of wild-type levels of RNA synthesis. The 5′ noncoding region of those revertants contained the mutation intact; mix-and-match experiments with the cDNA from these revertants revealed that a restricted region within the 3C gene was the site of the suppressing mutations in the revertants. The suppressors were point mutations, confirmed by introducing them into the 3C gene by site-directed mutagenesis. Although complementation studies indicated that the suppressors were *cis* active, we believe that protein changes rather than RNA sequence alterations are responsible for the suppression because RNA changes that did not alter protein sequence had no effect, whereas various protein alterations were suppressive. The results therefore imply that protein 3C interacts with the 5′ end of the RNA and may play a role in RNA replication.

Poliovirus, a member of the picornavirus family, contains a single plus-strand RNA genome of approximately 7.5 kilobases. There are two noncoding regions, at the 5′ and 3′ ends, flanking a translated region of 6,528 nucleotides (4, 13). Poliovirus RNA is translated into a single polypeptide, which is processed into its smaller, functional proteins mainly by the viral protease $3C^{pro}$ with a few cleavages by $2A^{pro}$ (2, 19).

The 5′ noncoding (5′NC) region shows marked sequence and secondary structure conservation among the three poliovirus serotypes, suggesting that the 5′-terminal region mediates crucial functions in the viral life cycle (14). We (20) and others (12) have been able to delineate at least two functional regions, one somehow involved in the synthesis of the viral RNA (the first 100 nucleotides) and the other involved in RNA translation (region P).

In an attempt to identify viral proteins that might interact with the 5′NC segment of the RNA, we isolated revertants from a mutant (5NC-11) that has a 4-base insertion at nucleotide 70 and is deficient in RNA synthesis (20). These revertants result from second site suppressor mutations within the $3C^{pro}$ gene. The existence of such suppressors strongly suggests a direct interaction between $3C^{pro}$ and the far end of the 5′NC. $3C^{pro}$ may therefore play an unexpected role in RNA replication.

MATERIALS AND METHODS

DNA procedures. Restriction enzymes, T4 DNA polymerase, T4 DNA ligase, DNA polymerase I (Klenow fragment), and human placenta RNase inhibitor were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind.; avian myeloblastosis virus reverse transcriptase was from Life Science Research, Baltimore, Md. All enzymes and compounds were used according to the instructions of the manufacturers. pGEM plasmids were from Promega Biotec Co., Madison, Wis.

Standard cloning techniques were used (8). Plasmid pPN-3, containing a mutation at nucleotide position 70, has been described previously (20).

The cDNA synthesis and cloning have been described in detail elsewhere (8). Briefly, viral RNA obtained from CsCl-purified virions was incubated with avian myeloblastosis virus reverse transcriptase and oligo(dT). The second-strand synthesis was performed by incubation of the heteroduplex (viral RNA first strand) with RNase H and T4 DNA polymerase; the double-stranded cDNA synthesized was digested with *Bgl*II and cloned into a vector (pGEM-3 based) containing the *Bgl*II site at the polylinker sequence, between *Bgl*II and *Nru*I sites. We generated two plasmids: p3A5′, containing a partial P1 region and a complete P2 and P3 until the *Bgl*II site at position 5601; and p3A3′, carrying cDNA from the *Bgl*II site (position 5601) in the 3C gene to the poly(A) (3′ end).

Cells and virus. HeLa cells were grown as previously described (1). HeLa cells on 100-mm dishes were transfected with 1 to 5 μg of in vitro-synthesized RNA by using the DEAE-dextran procedure (7). Individual plaques isolated from transfection were grown by standard procedures, and these stocks were used to analyze phenotypes.

Measurement of RNA synthesis. Suspension cultures of HeLa cells (about 10^7 cells per ml) were infected at a multiplicity of infection of 10. After adsorption at room temperature for 30 min, Dulbecco modified Eagle medium supplemented with 7% fetal calf serum was added. At various times after infection cells were placed on ice, washed once with cold phosphate-buffered saline, and lysed in 10 mM Tris hydrochloride (pH 7.5)–10 mM NaCl–0.1% Nonidet P-40. Nuclei and debris were removed by centrifugation. RNA was isolated by phenol-chloroform extraction and ethanol precipitation. Portions were denatured in 10× SSC (1× SSC is 0.15 M NaCl–15 mM sodium citrate)–17% formaldehyde and bound to nitrocellulose by aspiration with a dot blot apparatus. Filters were baked for 2 h at 80°C under vacuum. Hybridization was performed as described previously (20) with an antisense [^{32}P]RNA probe generated with T7 polymerase (9).

Nucleotide sequencing of RNA. Total cytoplasmic RNA from infected HeLa cells was prepared as described above and was sequenced by the chain-termination technique (15) by using reverse transcriptase and synthetic oligonucleotide

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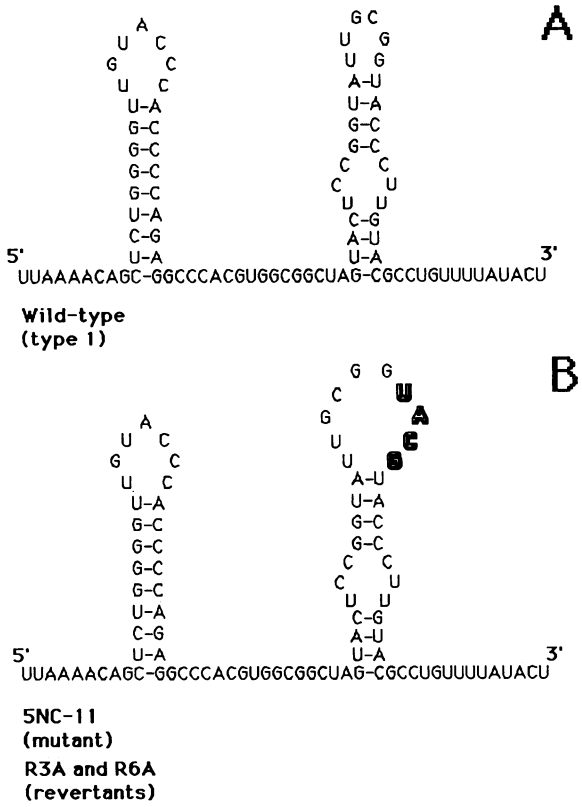

FIG. 1. Sequence at the 5'NC end of poliovirus RNA: (A) wild type (type I, Mahoney) and (B) mutant 5NC-11. The sequences determined by chain termination technique are displayed with proposed secondary structures. The 4-base insertion in 5NC-11 is indicated in larger type.

primers complementary to bases 102 to 124 and 6024 to 6045 of the viral RNA.

Site-directed mutagenesis. M13-rP3 was constructed for mutagenesis of the 3C gene. Briefly, DNA from plasmid pT7 (containing a complete sequence of poliovirus type 1 [Mahoney] cDNA, downstream of a T7 RNA polymerase promoter) was digested with *Bam*HI and *Eco*RI, and a resulting 2,900-base-pair fragment (encoding the complete P3 region) was electrophoretically purified. It was then cloned into the *Bam*HI-*Eco*RI sites of the replicative form of M13 mp19. The recombinant M13 contained poliovirus plus-sense sequences. Mutagenesis was carried out as described previously with uracil-enriched templates and specific mutated oligonucleotides primers complementary to poliovirus RNA sequences (5). Bacteriophages containing desired mutations were plaque purified. The poliovirus-specific fragment was excised from M13 replicative form by digestion with *Bgl*II and *Cla*I and electrophoretically purified. This fragment was then used as a substitute for the corresponding wild-type fragment in pT7 or pPN-3 (20).

RESULTS

Mutant 5NC-11 and its revertants. The mutant 5NC-11 (20) was generated by duplication of 4 bases near nucleotide position 70 at the far 5' end of the genome, where two very well conserved RNA loops are predicted by computational analysis (14) (Fig. 1). This insertion produced a virus that yielded minute plaques (Fig. 2B) and that was temperature sensitive (the titer at 32°C was 200-fold that at 39°C). The mutant had a dramatic deficiency in RNA synthesis (20) (Table 1).

Revertants of this mutant were spontaneously generated as large-plaque variants after one or two passages in HeLa cells at 37°C (Fig. 2B and C). Two of those revertants, R3A and R6A, were picked and grown up as stocks (their plaques are shown in Fig. 2D and E).

R3A was still somewhat deficient in progeny production and showed optimal growth at 37°C. R6A was even more deficient, although much better than the 5NC-11 mutant, and also had a 37°C optimum (data not shown).

Viral RNA and protein synthesis. Because mutant 5NC-11 has a major defect in RNA synthesis, we analyzed the ability of the revertants to produce positive-strand viral RNA by dot blotting. 5NC-11 produced only 0.1% of the wild-type amount of RNA (Table 1), whereas R3A made 62% and R6A produced 8.0%, both measured at their optimum temperature of 37°C. Both revertants showed cold and heat sensitivity of RNA synthesis, suggesting that their ability to produce progeny was limited by their ability to make RNA under all conditions.

Pulse-labeling experiments were performed to study the ability of these revertants to synthesize and process viral proteins. Overall protein synthesis was lower than that in the wild type, probably as a consequence of the lower amount of RNA template, but there was no evidence of any change in the proteolytic digestion pattern (data not shown).

Location of suppressors. Total cytoplasmic RNA from HeLa cells infected with the revertants was prepared, and the nucleotide sequence from 1 to 100 was determined. The very 5'NC region from R3A and R6A turned out to be identical to that carried by the original mutant (5NC-11) (Fig. 1). To map the element responsible for the revertant phenotype, genomic RNA from purified R3A viral particles was used as a template to synthesize cDNA. The cDNA was cloned into a pGEM-3-derived plasmid, generating two different plasmids, p3A3' and p3AP2 (see Materials and Methods).

A series of cDNA recombinants between the plasmid containing the insertion at nucleotide 70 (pPN-3) and different R3A cDNA fragments was constructed (Fig. 3), and the phenotypes of the resulting viruses were tested by plaque assay. Plasmid pNB-2 contained a full-length poliovirus cDNA, in which part of P1, all of P2, and a part of the P3 regions came from R3A cDNA (Fig. 3). When RNA derived from pNB-2 was transfected into HeLa cells, the plaque size of the resulting virus resembled that of the 5NC-11 mutant. By contrast, replacement of the wild-type 3' end with cDNA from the revertant generated a plasmid, pB3'end, whose RNA transcript produced the plaque size of the R3A revertant virus when transfected into HeLa cells. Other plasmids mapped the suppressing locus to a fragment extending from nucleotide positions 5601 to 6056 of the R3A genome (Fig. 3). When this 455-base-pair cDNA segment was sequenced, a single substitution, C to T at nucleotide position 5898, was found within the gene $3C^{pro}$ (the viral protease). Sequencing of R6A RNA revealed a different single base change also in the $3C^{pro}$ gene, 6 nucleotides downstream from the R3A substitution (Fig. 4).

To confirm that those changes really suppressed the 5NC-11 defect, we produced the same alterations directly in the mutant genome. Synthetic oligonucleotide primers were used to change C_{5898} to T (p11T) and A_{5904} to G (p11G) (see Materials and Methods) (Fig. 5). When RNA derived from

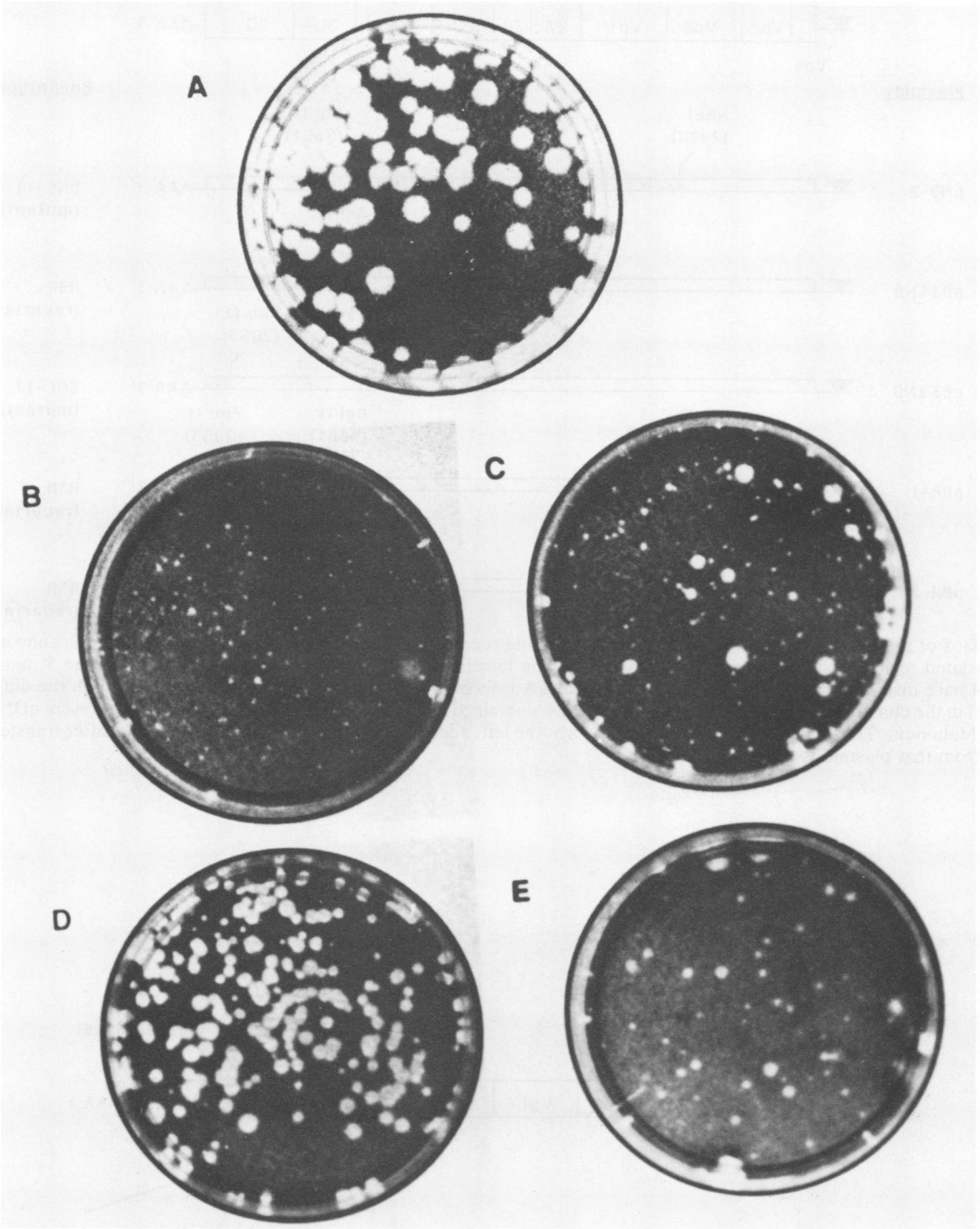

FIG. 2. Phenotype of mutant 5NC-11 and its revertants. In the plaque assay shown, HeLa cells were inoculated with the wild type, mutant, or revertants from master stocks, incubated at 37°C for approximately 40 h, and then stained. (A) Wild type; (B and C) mutant 5NC-11, first and second passage, respectively (the larger plaques are spontaneous revertants); (D) revertant R3A; (E) revertant R6A.

p11T and p11G was used to transfect HeLa cells, the viruses recovered had the plaque sizes of R3A and R6A, respectively (Fig. 5). Thus, independent mutated cDNAs obtained in this manner were able to reproduce exactly the phenotypes observed in R3A and R6A revertants (as assessed by plaque size), supporting the idea that C_{5898} to T and A_{5904} to G are the alterations needed to suppress the defect present in 5NC-11.

When the 3C alterations in R3A and R6A were inserted into a wild-type background, the Thr-to-Ile change in R3A had a plaque size approximately that of R3A, whereas the

TABLE 1. Synthesis of positive-strand poliovirus RNA

Virus	% of wild-type RNA synthesis		
	32°C (7 h)[a]	37°C (4 h)	39°C (4 h)
5NC-11	ND[b]	1.1	ND
R3A	37	62.0	9.7
R6A	2.8	8.0	2.1

[a] Numbers within parentheses indicate hours postinfection.
[b] ND, Not determined.

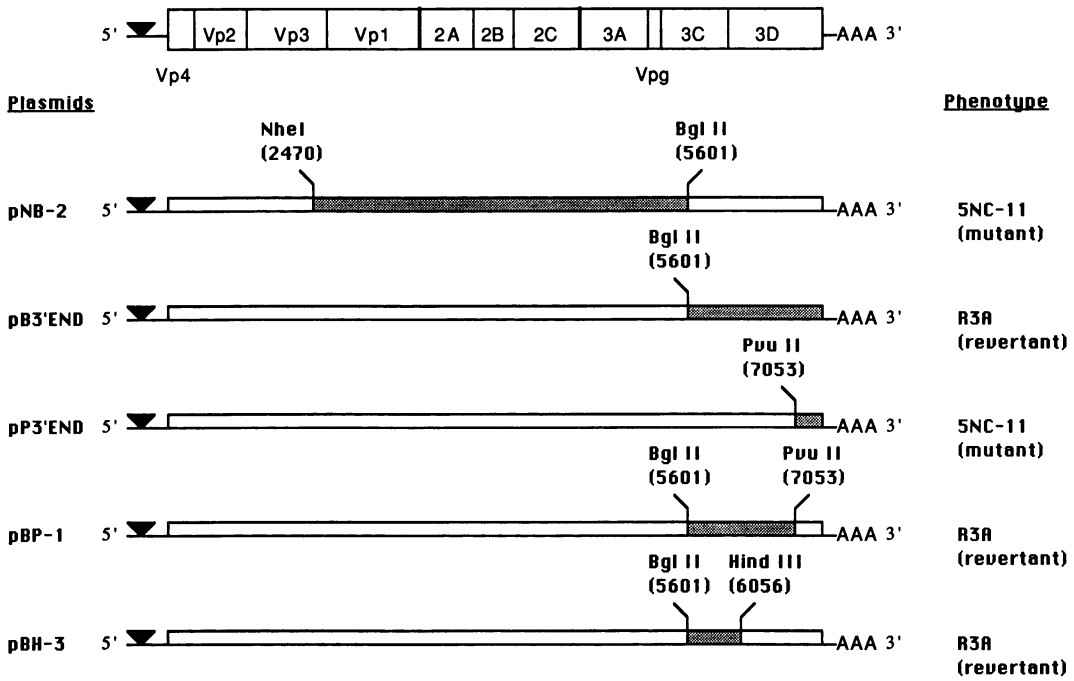

FIG. 3. Maps of full-length poliovirus cDNAs used to generate recombinant virus. The poliovirus genetic map is shown at the top (thin line, nontranslated regions; boxes, open reading frame with the location of the viral proteins). The triangles at the 5′ untranslated region indicate the 4-base insertion at position 70 (Fig. 1B). The cDNA inserts of different plasmids are shown below with the different restriction enzymes used in the constructions. Stippled bars indicate cDNA obtained from R3A revertant RNA; clear bars represent cDNA from the wild type, type I (Mahoney). The name of each plasmid is shown at the left, and the plaque assay phenotype obtained after transfection with RNA synthesized from that plasmid is shown at the right.

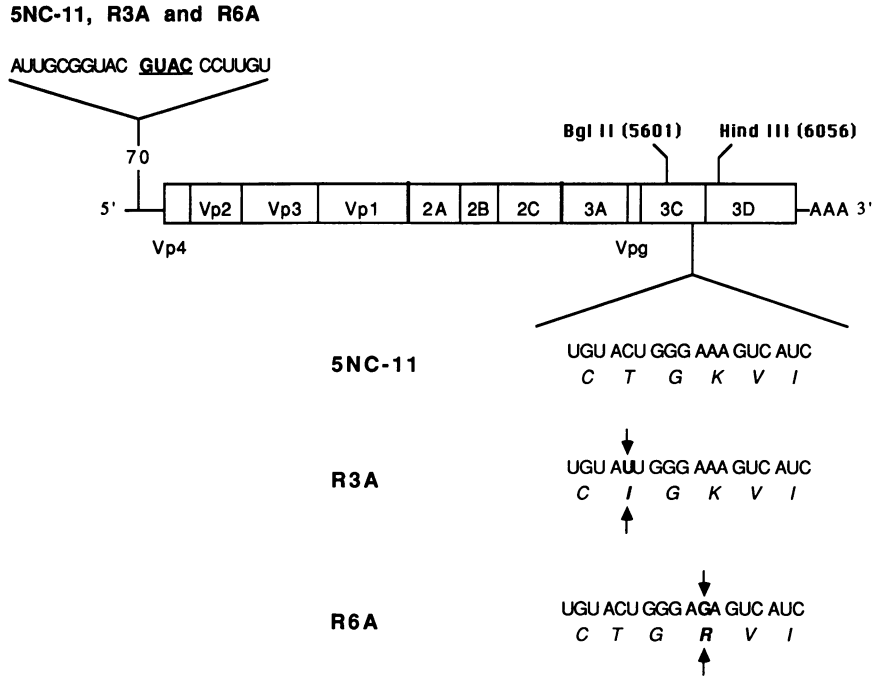

FIG. 4. Nucleotide sequence for 5NC-11, R3A, and R6A at the 5′ untranslated region and the $3C^{pro}$ gene. The restriction enzyme sites flanking the 3C-3D fragment (see the text) are indicated. The nucleotide and amino acid changes are indicated by boldface characters and arrows.

PLASMID		PHENOTYPE
pT7-polio	UGU ACU GGG AAA GUC AUC C T G K V I	Wild-type
pGTG XpA	UGU ACG GGU AAG GUC AUC C T G K V I	Wild-type
p11T	UGU AUU GGG AAA GUC AUC C I G K V I	R3A
p11TA	UGU AUA GGG AAA GUC AUC C I G K V I	R3A
p11G	UGU ACA GGG AGA GUC AUC C T G R V I	R6A
p11GCG	UGU ACA GGG CGC GUC AUC C T G R V I	R6A

FIG. 5. Site-specific mutagenesis. The sequence at the $3C^{pro}$ gene is shown. Changes that have been introduced are indicated in larger type. The name of each plasmid is shown at the left, and the phenotype of the virus generated after transfection with the RNA made from that plasmid is shown at the right. For all plasmids the first base of the sequence is 5894, and the amino acid coded for by the first codon is no. 153 of the 3C protein.

conservative Lys-to-Arg change in R6A generated a virus with a plaque size about equal to that of the wild type. Furthermore, the R3A lesion generated a virus that produced a slightly higher titer than did R3A as seen in a one-step growth curve. However, the same virus showed no difference from R3A with respect to the amount of viral RNA and protein synthesized, nor did it differ in terms of the processing of the polyprotein into its final products.

Silent mutation still conserves the phenotype. The suppressor of the 5NC-11 could either work through protein or directly by RNA-RNA interaction. We attempted to use complementation analysis, but the result yielded no information because the function appeared to be *cis* active (data not shown). Thus, to determine whether it was the $3C^{pro}$ protein or the RNA itself of this region that had the suppressor function, we generated silent mutations by changing particular codons to different ones encoding the same amino acid (Fig. 5). A clone with three silent changes in the region of $3C^{pro}$ where the suppressor mutations occurred was wild type, indicating that conservation of RNA structure in this region is not crucial.

Furthermore, when codon ATT_{154} of the R3A $3C^{pro}$ gene was substituted by ATA in the plasmid containing the 4-base insertion at the position 70 (Fig. 5; p11TA), the change, which did not modify the amino acid sequence, did not alter the phenotype of the virus generated. Similarly, when codon AGA_{156} of the R6A $3C^{pro}$ gene was replaced by CGC, a mutation which conserves the R6A 3C protein sequence, we recovered virus with the R6A phenotype.

Thus, although the data is fragmentary, they suggest that it is protein 3C rather than the RNA that carries out the suppressor function of the R3A and R6A revertants. Also, the various poliovirus types show variety in the RNA sequence in this region, indicating no tendency to maintain any particular RNA structure (10, 16–18).

VIRUS:	$3C^{PRO}$ SEQUENCES
POLIOVIRUS, TYPE I (MAHONEY)	...AUC ACA UGU ACU GGG AAA UCA UCG GGA... ...I T C T G K S S G...
POLIOVIRUS, TYPE II (LANSING)	... AUC ACC UGC ACU GGC AAG GUC AUC GGG... ...I T C T G K V I G...
COXSACKIEVIRUS B3	...CUC AUG UCC ACC GGC AAG GUA CUG GGU... ...L M S T G K V L G...
RHINOVIRUS 14	...CUG UGU GCU ACU GGU AAG AUC UUU GGU... ...L C A T G K I F G...

FIG. 6. Alignment of poliovirus types I and II, coxsackievirus B3, and human rhinovirus 14 sequences at the $3C^{pro}$ gene.

DISCUSSION

Starting with a mutant in the far 5′ untranslated segment of poliovirus RNA, we isolated revertants that, surprisingly, mapped to a protease gene, $3C^{pro}$. Two observations suggest that it is the protein 3C or one of its precursors, rather than the RNA sequences encoding 3C, that has the ability to suppress the 5NC-11 mutation. First, mutations affecting 3C RNA sequence but not the protein did not modify the phenotype of either the wild type or the revertants (Fig. 5). Second, in this particular region of 3C, the amino acid sequence (Thr-Gly-Lys) is highly conserved among different strains of poliovirus and other picornaviruses, whereas the RNA sequences differ significantly (Fig. 6). Interestingly, intertypic recombinant viruses between poliovirus types I and II (6) and between poliovirus and coxsackievirus (3) have been constructed. These recombinants, carrying 5′NC regions from one virus and 3C from another, generate wild-type virus, showing no indication of a specific interaction between the RNAs of the 5′NC region and the 3C region.

Thus, substitutions at the 3C protein appear to compensate for a mutation in the 5′NC region which could only affect RNA structure. This observation suggests strongly that a protein carrying 3C sequences must interact physically with RNA structures within the 5′NC region and that this interaction would be somehow affected by the mutation at nucleotide 70. We imagine that the mutationally altered interaction between 5′NC RNA structure and protein 3C is returned toward normal by readapting 3C to the mutated binding site. This readaptation of 3C can occur by means of the secondary mutations in the revertants R3A and R6A.

To interpret the meaning of the proposed interaction between protein 3C and the region around nucleotide 70 of the viral RNA, we need to understand the physiologic consequences of the lesion in mutant 5NC-11. This is a highly defective virus, yielding few progeny even at low temperature. It makes little viral RNA, and that might be its primary defect. However, viral RNA synthesis requires formation of correct viral proteins, implying that 5NC-11 could either be mutant in viral RNA synthesis directly or in formation of the active components of the RNA replication machinery. For instance, a defect in cleavage of the polyprotein precursor of the functional polypeptides could give an equivalent phenotype. Because 5NC-11 reverts easily and the revertants then go through a normal life cycle, obscuring the products of 5NC-11, we are uncertain exactly what is the physiologic consequence of the insertion in the mutant.

If 5NC-11 does have a defect in protein cleavage, then the data would indicate the $3C^{pro}$ requires an interaction with the 5′ end of the RNA for it to be appropriately active. This

would readily explain the data and would suggest that viral RNA is a cofactor of protease action. The 3C protease translated from an RNA lacking the poliovirus 5'NC sequence, however, carries out at least some cleavages (11), making the hypothesis unlikely. More likely is that the 3C region of polypeptide has a critical function in viral RNA synthesis that is manifest through its binding to the stem-loop region around nucleotide 70 of the 5'NC region. One possible function for 3C in replication relates to the VPg protein on the 5' end of viral RNA. This protein is mainly found in the cell as a precursor (3AB) from which it apparently cleaved at a Gln-Gly bond, the primary substrate for $3C^{pro}$ cleavage. Thus, $3C^{pro}$ may bind into the initiation complex to coordinate VPg cleavage from 3AB, and the structure around nucleotide 70 may provide the binding site. We are presently attempting to derive alleles of $3C^{pro}$ or 5'NC that would be more amenable to physiologic analysis to test these possibilities.

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